Potentiation of NMDA Receptor-Mediated Responses by Dynorphin at Low Extracellular Glycine Concentrations

LI ZHANG, ROBERT W. PEOPLES, MURAT OZ, JUDITH HARVEY-WHITE, FORREST F. WEIGHT, AND ULRIKE BRAUNEIS

Laboratory of Molecular and Cellular Neurobiology, National Institute on Alcohol Abuse and Alcoholism, and Clinical Neuroscience Branch, National Institute of Neurological Disorders and Stroke, National Institutes of Health, Bethesda, Maryland 20892

Zhang, Li, Robert W. Peoples, Murat Oz, Judith Harvey-White, Forrest F. Weight, and Ulrike Brauneis. Potentiation of NMDA receptor-mediated responses by dynorphin at low extracellular glycine concentrations. *J. Neurophysiol.* 78: 582–590, 1997. The effect of dynorphin A(1–13) on *N*-methyl-D-aspartate (NMDA)-activated currents was investigated in the presence of low extracellular glycine concentrations in *Xenopus* oocytes expressing recombinant heteromeric NMDA receptors and in cultured hippocampal neurons with the use of voltage-clamp techniques. At an extracellular added glycine concentration of 100 nM, dynorphin A(1–13) (10 μM) greatly increased the amplitude of NMDA-activated currents for all heteromeric subunit combinations tested; on average, the potentiation was: $\epsilon 1/\zeta 1$, 3,377 ± 1,416% (mean ± SE); $\epsilon 2/\zeta 1$, 1,897 ± 893%; $\epsilon 3/\zeta 1$, 4,356 ± 846%; and $\epsilon 4/\zeta 1$, 1,783 ± 503%. Potentiation of NMDA-activated current by dynorphin A(1–13) was concentration dependent between 0.1 and 10 μM dynorphin A(1–13), with a half-maximal concentration value of 2.77 μM and an apparent Hill coefficient of 2.53, for $\epsilon 2/\zeta 1$ subunits at 100 nM added extracellular glycine. Percentage potentiation by dynorphin A(1–13) was maximal at the lowest glycine concentrations tested (0.01 and 0.1 μM), and decreased with increasing glycine concentration. No significant potentiation was observed at glycine concentrations >0.1 μM for $\epsilon 1/\zeta 1$, $\epsilon 2/\zeta 1$, and $\epsilon 4/\zeta 1$ subunits, or at >1 μM for $\epsilon 3/\zeta 1$ subunits. Potentiation of NMDA-activated currents by dynorphin A(1–13) was not inhibited by 1 μM of the κ-opioid receptor antagonist nor-binaltorphimine, and potentiation was not observed with 10 μM of the κ-opioid receptor agonist *trans*-3,4-dichloro-*N*-methyl-*N*-[2-(1-pyrrolidinyl)-cyclohexyl]benzene-acetamide. Potentiation of NMDA-activated current by dynorphin A(1–13) was inhibited by the glycine antagonist kynurenic acid (50 μM). NMDA-activated current was also potentiated at low glycine concentrations by 10 μM dynorphin A(2–13) or (3–13), both of which have a glycine as the first amino acid, but not by 10 μM dynorphin A(4–13), which does not have glycine as an amino acid. In hippocampal neurons, 10 μM dynorphin A(1–13) or (2–13) potentiated steady-state NMDA-activated current in the absence of added extracellular glycine. The extracellular free glycine concentration, determined by high-performance liquid chromatography, was between 26 and 36 nM for the bathing solution in presence or absence of 10 μM dynorphin A(1–13), (2–13), (3–13), or (4–13), and did not differ significantly among these solutions. The observations are consistent with the potentiation of NMDA-activated current at low extracellular glycine concentrations resulting from an interaction of the glycine amino acids in dynorphin A(1–13) with the glycine coagonist site on the NMDA receptor. Because dynorphin A is an endogenous peptide that can be coreleased with glutamate at glutamatergic synapses, the potentiation of NMDA receptor-mediated responses could be an important physiological regulator of NMDA receptor function at these synapses.

INTRODUCTION

Dynorphin opioids are a family of multiple structurally related peptides that derive from a common precursor, prodynorphin (Kakidani et al. 1982). Dynorphin A, a 17-amino-acid peptide, was first isolated from the porcine pituitary (Goldstein et al. 1979) and has been found in several locations in the spinal cord and brain: in laminae I and V of the dorsal horn (Cruz and Basbaum 1985), where it can be released in response to high-frequency stimulation (Hutchison et al. 1990); in the periaqueductal limbic system and the thalamus (Millan et al. 1984; Watson et al. 1982); in the adenohypophysis (Seizinger 1984); in dentate granule cells; and throughout the hippocampus (McGinty et al. 1983). High concentrations of immunoreactive dynorphin A have been demonstrated in the hippocampal mossy fiber pathway (McGinty et al. 1983; McLean et al. 1987), and dynorphin A was reported to be released from mossy fibers on stimulation of that pathway (Chavkin et al. 1983; Wagner et al. 1991) and coreleased with glutamate (Terrian et al. 1989).

As ligands of the κ-opioid receptors, the endogenous dynorphin A(1–17) (Weisskopf et al. 1993) and the synthetic dynorphin A(1–13) (Caudle and Isaac 1988; Chavkin et al. 1982) exert many of their effects via interaction with the κ_1 or κ_2 opioid receptors. But dynorphin A(1–17) (Bakshi and Faden 1990; Henriksen et al. 1982) and dynorphin A(1–13) (Skilling et al. 1992) also have nonopioid activity. It has been suggested that some of the actions of dynorphin A(1–17) (Dubner and Ruda 1992; Henriksen et al. 1982; Walker et al. 1982; Weisskopf et al. 1993) and dynorphin A(1–13) (Caudle and Isaac 1988) in the nervous system involve modulation of the *N*-methyl-D-aspartate (NMDA) subtype of glutamate receptors. Of these, inhibitory effects of dynorphin A(1–17) on NMDA receptors involve either the κ-opioid receptor, as with the inhibition of mossy fiber synaptic responses (Weisskopf et al. 1993) and of NMDA-mediated synaptic currents in CA3 pyramidal cells (Caudle et al. 1994), or a direct action on the NMDA receptor by dynorphin A(1–17) (Chen et al. 1995) or dynorphin A(1–13) (Massardier and Hunt 1989). In vivo, potentiating effects of dynorphin A on apparently NMDA receptor-medi-

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ated processes are, with a few exceptions (Moises and Walker 1985), nonopioid. For example, intrathecal injection of dynorphin A(1–17) (Bakshi and Faden 1990) or dynorphin A(1–13) (Skilling et al. 1992; Stevens et al. 1987) causes neurotoxicity, which results in hindlimb paralysis, and intracerebroventricular injection of dynorphin A(1–13) produces motor dysfunction, such as wild running and popcorn jumping (Shukla et al. 1992); these phenomena are prevented or reversed by treatment with NMDA receptor antagonists (Shukla et al. 1992; Skilling et al. 1992), suggesting that the action of dynorphin A(1–17) and dynorphin A(1–13) involves NMDA receptor-mediated responses. In contrast to these in vivo observations of potentiation of NMDA receptor-mediated effects, cellular electrophysiological studies have found mainly inhibitory effects of dynorphin A. For example, dynorphin A(1–13) and dynorphin A(2–13) were shown to depress spontaneous and glutamate-induced firing in single hippocampal CA1 and CA3 neurons (Walker et al. 1982). In addition, NMDA-activated currents were inhibited by dynorphin A(1–13), (1–17), and (1–32) in trigeminal neurons (Chen et al. 1995); by dynorphin A(1–17) in neurons from rat spinal dorsal horn (Cerne et al. 1994) and in CA3 pyramidal cells (Caudle et al. 1994); and by dynorphin A(1–13) in *Xenopus* oocytes expressing recombinant NMDA receptors (Brauneis et al. 1996b). One exception is a report of a potentiation of NMDA receptor-mediated synaptic currents at low concentrations of dynorphin A(1–17) in CA3 pyramidal cells (Caudle et al. 1994).

Some observations suggest that agents acting at the glycine coagonist site on NMDA receptors may affect the action of dynorphin on these receptors. For example, an antagonist at the glycine site on NMDA receptors, 7-chlorokynurenic acid, prevented dynorphin-A(1–17)-induced hindlimb paralysis (Bakshi and Faden 1990), and the glycine antagonist (±)HA-966 inhibited dynorphin A(1–13)-induced potentiation of the binding of the NMDA antagonist [^{3}H]CGP-39653 to rat brain membranes (Dumont and Lemaire 1994). These observations raised the question of whether the glycine coagonist site on NMDA receptors may be involved in the response of these receptors to dynorphin. We report here an investigation of the effect of extracellular glycine concentration on dynorphin A modulation of NMDA receptor function. For this work we used the synthetic, truncated dynorphin A(1–13), which constitutes the physiologically active part of dynorphin A(1–17) (Goldstein et al. 1979), to ensure continuity with our previous study (Brauneis et al. 1996b). The observations show that dynorphin A(1–13) can potentiate NMDA receptor-mediated responses at low extracellular glycine concentrations. Some of these results have been reported previously in preliminary form (Brauneis et al. 1995, 1996a).

METHODS

Molecular biology and oocyte expression

Mouse NMDA receptor subunit cDNA clones ζ1, ϵ1, ϵ2, ϵ3, and ϵ4 were provided by Dr. Masayoshi Mishina, Niigata University, Niigata, Japan. Subunit-specific cRNAs were synthesized from the corresponding cDNAs in vitro from linearized templates with the use of T3 RNA polymerase in the presence of 7-methyl-G cap analog. The cRNA combinations ϵ1/ζ1, ϵ2/ζ1, ϵ3/ζ1, or ϵ4/ζ1 (a total of 15 ng at molar proportions ϵ:ζ = 4:1) were injected into *Xenopus* oocytes with the use of a pressurized microinjection device (PV 800 Pneumatic Picopump, World Precision Instruments, Sarasota, FL). Mature *Xenopus laevis* frogs were anesthetized by immersion in water containing 3-aminobenzoic acid ethyl ester (2 g/l). Oocytes were excised and mechanically isolated into clusters of four to five oocytes, which were shaken in a water bath in two changes of 0.2% collagenase A (Sigma, St. Louis, MO) in a calcium-free bathing solution containing (in mM) 83 NaCl, 2 KCl, 1 $MgCl_2$, and 5 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), pH 7.5, for l h each, to remove the connective tissue and follicular layer. After injection with cRNA, oocytes were incubated for 2 days in modified Barth's saline (composition, in mM: 83 NaCl, 2 KCl, 1 $MgCl_2$, 2 $CaCl_2$, and 5 HEPES) containing sodium pyruvate (2 mM), penicillin (10,000 U/l), streptomycin (10 mg/l), gentamycin (50 mg/l), and theophylline (0.5 mM), all from Sigma.

Electrophysiology of oocytes

For electrophysiological recordings, oocytes were placed in a recording chamber (solution volume ~ 100 μl) and constantly superfused with bathing medium (composition, in mM: 95 NaCl, 2 KCl, 2 $BaCl_2$, and 5 HEPES, pH adjusted to 7.5 with NaOH) at a rate of ~2.5 ml/min. Membrane current was measured with the use of standard two-electrode voltage-clamp recording (Axoclamp-2, Axon Instruments, Foster City, CA) at a holding potential of −70 mV. Agonists and/or drugs were administered via the bathing solution, which was applied by gravity flow via a macropipette positioned ~3 mm from the oocyte. Usually, currents were activated by superfusion of the oocytes by agonist and/or agonist plus drug-containing bathing solution for 10–30 s. A washout time of ~5 min was allowed between successive agonist and/or agonist plus drug applications. Currents were recorded on a rectilinear pen recorder (Model 2400, Gould, Valley View, OH).

Culture of hippocampal neurons

Primary cultures of hippocampal neurons from 15- to 17-day fetal mice were prepared by a method modified from that of Goslin and Banker (1991). Briefly, hippocampi were dissected in Ca^{2+}- and Mg^{2+}-free Hank's balanced salt solution (HBSS; GIBCO BRL, Grand Island, NY) with 10 mM HEPES, incubated in 0.25% trypsin for 15 min, washed three times in HBSS, and mechanically triturated with the use of a fire-polished Pasteur pipette. The resulting cell suspension was then centrifuged and the cells were resuspended in HBSS. Neurons were plated on monolayers of hippocampal glia in 35-mm dishes in medium containing 90% minimal essential medium (GIBCO BRL), 10% heat-inactivated equine serum (HyClone, Logan, UT), 1 mM sodium pyruvate, and 0.004% DNase I (Boehringer Mannheim, Indianapolis, IN). After ~4 h, and subsequently once weekly, one-half of the medium was removed and replaced with maintenance medium consisting of minimal essential medium, 1 mM sodium pyruvate (Sigma), and N2 serum supplement (GIBCO BRL). Neurons were cultured for 1–4 wk before use in experiments.

Electrophysiology of hippocampal neurons

Patch-clamp recording of whole cell currents was performed in hippocampal neurons at 20–25°C with the use of an Axopatch 200 patch-clamp amplifier (Axon Instruments). Patch pipettes with tip resistances of 2–5 MΩ were used; series resistances of 3–10 MΩ were compensated by 80%. All recordings were performed in neurons voltage clamped at −50 mV. Solutions of NMDA and peptides were applied to neurons by gravity flow with the use of a linear

multibarrel array (diameter of each pipette ~300 μm) placed within 100 μm of the cell body to allow for rapid solution changes. Cells were constantly superfused by extracellular medium flowing from one pipette barrel (flow rate ~3 μl/s), and solutions were applied by opening a valve and moving the barrel array so that the desired solution bathed the cell. Solutions of NMDA and peptides were applied at intervals of ≥90 s to allow for full recovery from desensitization. The extracellular solution used in these experiments contained (in mM) 150 NaCl, 5 KCl, 1 $CaCl_2$, 10 HEPES, 10 glucose, and 0.0002 tetrodotoxin, pH adjusted to 7.4 with NaOH, osmolality adjusted to 340 mosmol/kg with sucrose. The patch pipette (internal) solution contained (in mM) 140 CsCl, 2 Mg_4ATP, 10 bis-(*o*-aminophenoxy)-*N*,*N*,*N*′,*N*′-tetraacetic acid, and 10 HEPES, pH adjusted to 7.4 with CsOH, osmolality adjusted to 310 mosmol/kg with sucrose. Solutions were prepared fresh daily.

Data analysis

Statistical analysis of concentration-response data was performed with the use of the nonlinear curve-fitting program ALLFIT (DeLean et al. 1978). Data were fitted to the logistic equation

$$y = \{(E_{max} - E_{min})/[1 + (x/EC_{50})^n]\} + E_{min}$$

where x and y are concentration and response, respectively; E_{max} and E_{min} are the maximal and minimal responses, respectively; EC_{50} is the half-maximal concentration; and n is the slope factor (apparent Hill coefficient). Data were statistically compared by the paired *t*-test, unpaired *t*-test, or analysis of variance (ANOVA), as noted. Average values are expressed as means ± SE.

Free glycine measurements

L-Methionine sulphone (30 μl of 0.5 μg in 0.1 M HCl, Sigma) was mixed with 100 μl of the solution to be tested or 100 μl of a standard solution (containing 1.0 μg/ml glycine) in a borosilicate glass culture tube (prewashed with 6 M HCl) and dried under nitrogen at 35°C (Multivap, Organomation Associates, Berlin, MA). The acid residue was neutralized with 10 μl of a drying solution, as described in the Waters' High-Performance Liquid Chromatography (HPLC) Operating Manual for PICO-TAG Amino Acid Analysis System (Manual #88140, Revision 4, Waters, Milford, MA). The samples were redried under nitrogen at 35°C. The samples were then derivatized and analyzed by HPLC with ultraviolet detection at 254 nm as described (Cohen et al. 1986), except that after derivatization excess reagent was removed by drying under nitrogen at 35°C for 60–80 min, and the residue was reconstituted in 27 μl PICO-TAG Diluent (Waters #88119) and 12 μl were injected. The Waters HPLC system consisted of two 510 pumps, Automated Gradient Controller Model 680, Temperature Control Module, Model 440 Absorbance Detector, and Rainin Mac Integrator II (Rainin, Woburn, MA). The HPLC conditions and quantification were as described for ''Analysis of physiological samples for free amino acids'' in the Waters PICO-TAG manual.

Sources of drugs and chemicals

NMDA, glycine, and dynorphin A(2–13) were purchased from Sigma; dynorphin A(1–13), (1–8), (1–17), and (1–32) were purchased from Peninsula Laboratories (Belmont, CA); dynorphin A(3–13) and (4–13) were custom synthesized by Peninsula Laboratories; kynurenic acid, nor-binaltorphimine (NorBN1), and *trans*-3,4-dichloro-*N*-methyl-*N*-[2-(1-pyrrolidinyl)-cyclohexyl]benzene-acetamide (U50488) were purchased from Research Biochemicals International (Natick, MA).

The care and use of animals in this study was approved by the Animal Care and Use Committee of the National Institute on Alcohol Abuse and Alcoholism in accordance with the National Institutes of Health Guidelines (protocol numbers LMCN-SP-01 and -05).

RESULTS

Effect of dynorphin A(1–13) on NMDA-activated currents at low glycine concentrations

Figure 1 illustrates the effect of 10 μM dynorphin A(1–13) on NMDA-activated currents in *Xenopus* oocytes expressing recombinant NMDA receptors at low extracellular glycine concentrations. In the presence of 100 nM added glycine, 100 μM NMDA elicited little current in oocytes expressing ε1/ζ1, ε2/ζ1, ε3/ζ1, or ε4/ζ1 subunits, as previously reported for glycine effects on glutamate-activated currents for recombinant NMDA receptors (Ikeda et al. 1994; Kutsuwada et al. 1992). The addition of 10 μM dynorphin A(1–13) to the NMDA-containing bathing solution increased the amplitude of NMDA-activated currents for each subunit combination by the following percentages: ε1/ζ1, 3,377 ± 1,416% (mean ± SE, $n = 13$); ε2/ζ1, 1,897 ± 893% ($n = 5$); ε3/ζ1, 4,356 ± 846% ($n = 12$); ε4/ζ1, 1,783 ± 503% ($n = 14$; $P < 0.05$, paired *t*-test). The potentiation of NMDA-activated current by dynorphin A(1–13) was fully reversible. To test whether dynorphin A(1–13) acted similarly to the endogenous dynorphin A(1–17) peptide, we determined the effect of 10 μM dynorphin A(1–

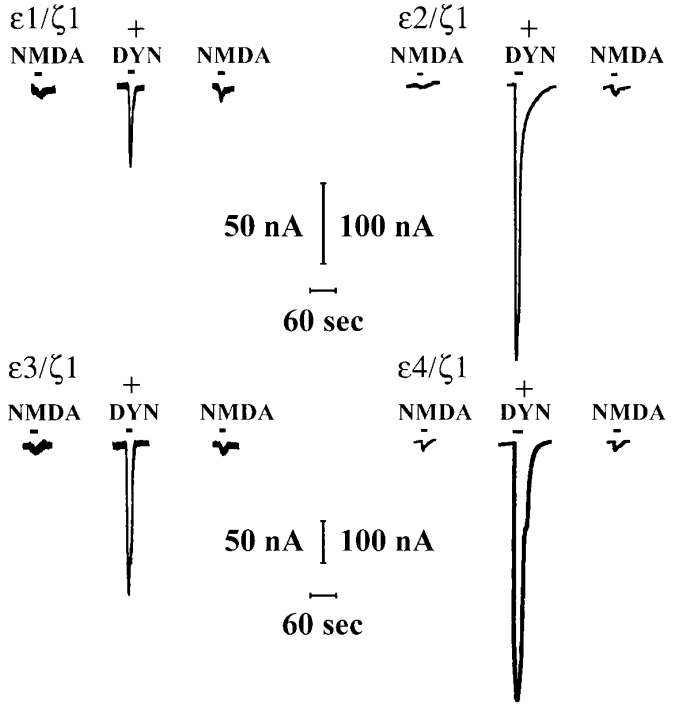

FIG. 1. Effect of dynorphin A(1–13) (DYN) on *N*-methyl-D-aspartate (NMDA)-activated currents at low extracellular glycine concentrations. Traces of representative currents, activated by 100 μM NMDA in presence of 100 nM added glycine for oocytes expressing ε1/ζ1, ε2/ζ1, ε3/ζ1, or ε4/ζ1 NMDA receptor subunits, as labeled. For each set, *left traces* are control currents, *middle traces* show effect of 10 μM dynorphin A(1–13) on NMDA-activated currents, and *right traces* are recovery ~5 min after application of dynorphin A(1–13).

17), as well as dynorphin A(1–8) and (1–32), on NMDA-activated current in the presence of 100 nM added glycine in oocytes expressing $\epsilon4/\zeta1$ subunits. Percentage potentiations (mean ± SE) for these peptides were, respectively, 857 ± 402% (n = 11), 1,150 ± 424% (n = 11), and 1,003 ± 360% (n = 10), with a control for dynorphin A(1–13) of 1,083 ± 415% (n = 11).

Concentration-response for dynorphin A(1–13) potentiation of NMDA-activated current

Figure 2*A* shows traces of currents activated by 100 μM NMDA in the presence of 100 nM added glycine and at the dynorphin A(1–13) concentrations indicated, recorded from an oocyte expressing $\epsilon2/\zeta1$ subunits. Note that NMDA activated little current at dynorphin concentrations of 0.01, 0.1, and 1 μM, whereas NMDA activated increasingly larger currents when dynorphin concentration was increased from 3 to 30 μM. Figure 2*B* is the average concentration-response curve for dynorphin potentiation of NMDA-activated current for oocytes expressing $\epsilon2/\zeta1$ subunits. Each point represents the average percentage potentiation (mean ± SE) of current activated by 100 μM NMDA in the presence of 100 nM glycine and at the dynorphin A(1–13) concentrations indicated. As can be seen, the potentiation of NMDA-activated current by dynorphin A(1–13) was concentration dependent between 0.1 and 10 μM dynorphin. The E_{max} of this concentration-response curve was 2,107% potentiation, the EC_{50} was 2.77 μM, and the apparent Hill coefficient was 2.53.

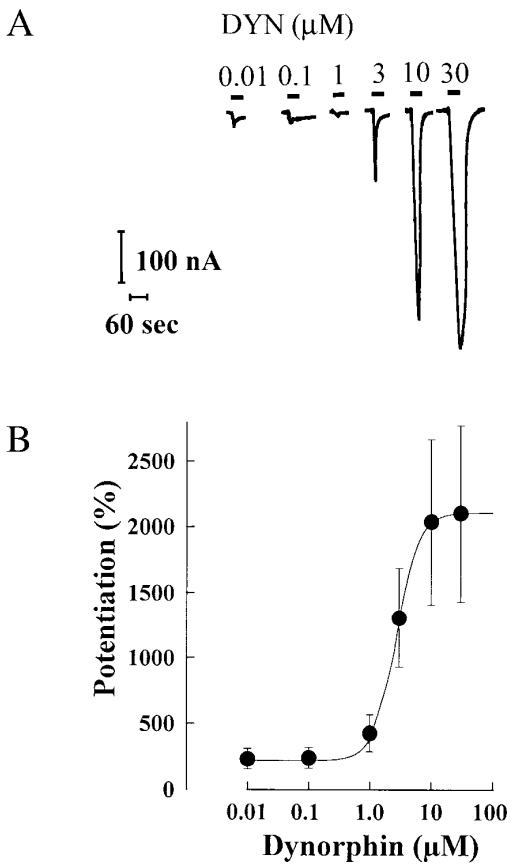

FIG. 2. Effect of dynorphin A(1–13) concentration on potentiation of NMDA-activated current in oocytes expressing $\epsilon2/\zeta1$ NMDA receptor subunits in presence of 100 nM added glycine. *A*: representative traces of NMDA-activated current in presence of dynorphin A(1–13) concentrations indicated, recorded from 1 oocyte. *B*: average percentage potentation of NMDA-activated current plotted as function of dynorphin A(1–13) concentration. Each data point represents mean ± SE from 5 oocytes.

Effect of glycine concentration on dynorphin A(1–13) potentiation of NMDA-activated current

Figure 3*A* shows the percentage potentiation of NMDA-activated current by dynorphin A(1–13) plotted as a function of added glycine concentration for the heteromeric NMDA subunit combinations. Fitting the data to the logistic equation yielded the following respective E_{max} values: $\epsilon1/\zeta1$, 2,697%; $\epsilon2/\zeta1$, 1,927%; $\epsilon3/\zeta1$, 3,737%; and $\epsilon4/\zeta1$, 1,411%. For all subunit combinations, 10 μM dynorphin A(1–13) potentiated NMDA-activated current significantly at added glycine concentrations of 10 and 100 nM, and at an added glycine concentration of 1 μM as well for $\epsilon3/\zeta1$ ($P < 0.05$, ANOVA). As the glycine concentration was increased, the dynorphin potentiation of NMDA-activated current decreased. The fitted curves indicate that the magnitude of the dynorphin potentiation decreased by half at the following glycine concentrations: $\epsilon1/\zeta1$, 0.24 μM; $\epsilon2/\zeta1$, 2 μM; $\epsilon3/\zeta1$, 0.47 μM; and $\epsilon4/\zeta1$, 0.93 μM. Dynorphin A(1–13), in the absence of NMDA, did not activate detectable current, but a dynorphin-induced potentiation of NMDA-activated current was observed in the absence of added glycine [238 ± 55 nA in the presence of 10 μM dynorphin A(1–13) vs. 5 ± 1 nA in the absence of dynorphin A(1–13); $n = 7$, $P < 0.005$, paired t-test]. Figure 3*B* shows the effect of glycine concentration on the amplitude of NMDA-activated current in the absence and presence of dynorphin A(1–13) (10 μM). The highest glycine concentration tested was 10 μM, which has been reported to be a maximal concentration for glycine potentiation of NMDA- or glutamate-activated currents (Henderson et al. 1990). Assuming that 10 μM was also maximal for glycine potentiation of NMDA-activated current in the experiments reported here, dynorphin A(1–13) (10 μM) did not significantly alter the EC_{50} values of the glycine concentration-response curves. The respective EC_{50} values in the absence and presence of dynorphin A(1–13) (10 μM) were as follows: $\epsilon1/\zeta1$, 2.13 versus 1.51 μM; $\epsilon2/\zeta1$, 1.32 versus 2.38 μM; $\epsilon3/\zeta1$, 2.13 versus 3.89 μM; and $\epsilon4/\zeta1$, 0.54 versus 1.07 μM ($P > 0.05$, ANOVA). Note also that in Fig. 3*B* the variability of current amplitude in the presence of dynorphin A(1–13) was relatively small, whereas in Fig. 3*A* the variability in percentage potentiation by dynorphin A(1–13) was quite large. This large variability was caused by a considerable difference between individual oocytes in the amplitude of NMDA-activated current at low glycine concentrations in the absence of dynorphin A(1–13). For example, in oocytes expressing $\epsilon2/\zeta1$ subunits, NMDA-activated current in the presence of 10 μM dynorphin A(1–13) might be 500 nA in one oocyte and 510 nA in another; however, if the control NMDA-activated current was 0.5 nA in the first oocyte and 0.17 nA in the second oocyte, the percentage potentiation would be 1,000 and 3,000%, respectively.

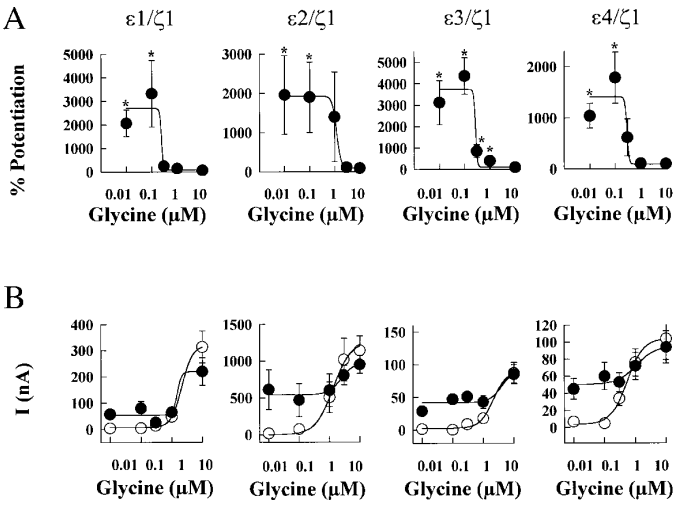

FIG. 3. Effect of glycine concentration on potentiation of NMDA-activated current by dynorphin A(1–13) (10 μM). *A*: percentage potentiation of NMDA-activated currents at added glycine concentrations indicated for oocytes expressing $\epsilon1/\zeta1$, $\epsilon2/\zeta1$, $\epsilon3/\zeta1$, or $\epsilon4/\zeta1$ NMDA receptor subunits. Asterisks: significant potentiation [$P < 0.05$, analysis of variance (ANOVA)]. *B*: glycine concentration-response curves for NMDA-activated currents in absence (○) and presence (●) of 10 μM dynorphin A(1–13) for oocytes expressing $\epsilon1/\zeta1$, $\epsilon2/\zeta1$, $\epsilon3/\zeta1$, or $\epsilon4/\zeta1$ NMDA receptor subunits. In *A* and *B*, each data point represents mean ± SE for 6–12 oocytes. Error bars not visible are smaller than symbol. Curves shown are fitted to logistic equation given in METHODS.

Effect of κ-opioid receptor agonist and antagonist on dynorphin A(1–13) potentiation of NMDA-activated current

Dynorphin A exerts many of its effects by interaction with κ-opioid receptors. To determine whether the potentiation of NMDA receptor-mediated responses by dynorphin A(1–13) involves κ-opioid receptors, we studied the effect of the κ-opioid receptor agonist U50488 and the κ-opioid receptor antagonist NorBN1. Figure 4*A* shows traces recorded from an individual oocyte in the presence of 100 nM added glycine, illustrating that there was little current when either NMDA, 10 μM U50488 alone, or 10 μM U50488 plus NMDA was applied. The current was greatly increased in amplitude when 10 μM dynorphin A(1–13) was applied with NMDA in the same oocyte. On average, in the presence of 100 nM added glycine, there was no significant difference between currents activated by NMDA, 10 μM U50488, or NMDA plus 10 μM U50488 (Fig. 4*B; P* > 0.05, ANOVA). Figure 4*C* illustrates the effect of NorBN1 on NMDA-activated currents. In the presence of 100 nM added glycine, NMDA activated little current in the absence or presence of 3 μM NorBN1. Dynorphin A(1–13) (10 μM), in contrast, potentiated NMDA-activated current, and this potentiation was not inhibited by 1 μM NorBN1. There was no significant difference between the effect of NMDA in the absence or presence of 3 μM NorBN1, or between the dynorphin A(1–13) potentiation of NMDA-activated current in the absence or presence of NorBN1 (Fig. 4*D; P* > 0.05, paired *t*-test).

Effect of a glycine antagonist on dynorphin A(1–13) potentiation of NMDA-activated current

The properties of the dynorphin A(1–13) potentiation of NMDA-activated current suggested that dynorphin A(1–13) may have a glycinelike action on NMDA receptors. We therefore tested the effect of kynurenic acid, a competitive antagonist at the glycine coagonist site on NMDA receptors. Figure 5*A* illustrates representative current records of the potentiation of NMDA-activated current by 10 μM dynorphin A(1–13) and in the presence of 100 nM added glycine, and the inhibition of this potentiation by 50 μM kynurenic acid in an oocyte expressing the $\epsilon4/\zeta1$ subunits. The bar graphs in Fig. 5*B* show the average data from five oocytes. Potentiation of NMDA-activated current by 10 μM dynorphin A(1–13), expressed as percentage of control, was 7,727 ± 4,142%, and this potentiation was significantly inhibited to 1,561 ± 1,385% in the presence of 50 μM kynurenic acid ($P < 0.005$, paired *t*-test).

Effect of dynorphin A(1–13), (2–13), (3–13), and (4–13) on NMDA-activated current at low glycine concentrations

The first amino acid of the dynorphin A peptide, a tyrosine, has been found to be essential for the κ-opioid effects

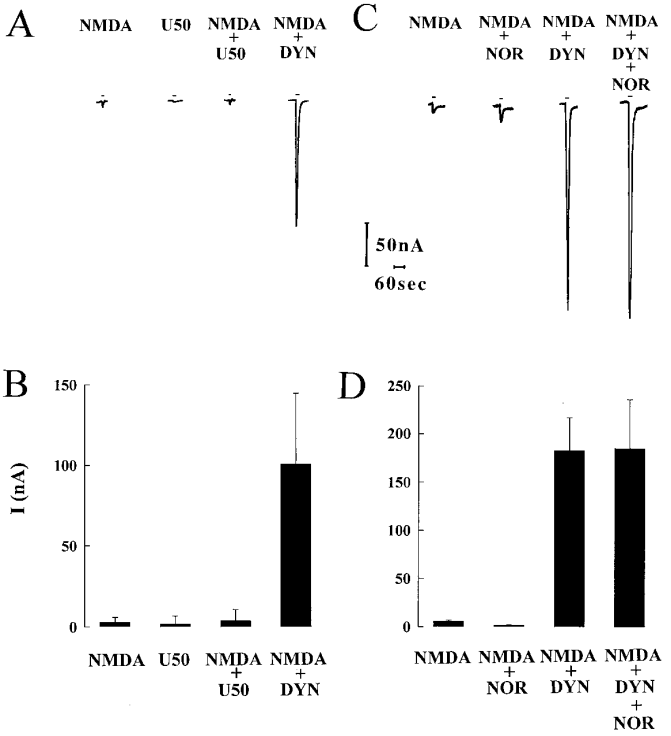

FIG. 4. Effects of κ-opioid agonist and antagonist on NMDA-activated current and dynorphin A(1–13) potentiation of NMDA-activated current. *A*: representative traces showing effect of κ-opioid agonist *trans*-3,4-dichloro-*N*-methyl-*N*-[2-(1-pyrrolidinyl)-cyclohexyl]benzene-acetamide (U50488, 10 μM) recorded from oocyte expressing $\epsilon4/\zeta1$ NMDA receptor subunits. From *left* to *right*, *1st trace* shows control NMDA-activated current, *2nd trace* shows that U50488 alone elicited no appreciable current, and *3rd trace* shows that U50488 did not potentiate NMDA-activated current, unlike 10 μM dynorphin A(1–13) (*4th trace*). *B*: bar graphs showing average (mean ± SE) data relevant to traces in *A* ($n = 6$) *C*: representative traces showing effect of the κ-opioid antagonist nor-binaltorphimine (NorBN1) recorded from oocyte expressing $\epsilon4/\zeta1$. From *left* to *right*, amplitude of NMDA-activated current (*trace 1*) was not changed by presence of 3 μM NorBN1 (*trace 2*), nor was potentiation of NMDA-activated current by 10 μM dynorphin A(1–13) (*trace 3*) inhibited by 1 μM NorBN1 (*trace 4*). *D*: bar graphs showing average (mean ± SE) data relevant to traces in *C* ($n = 6$).

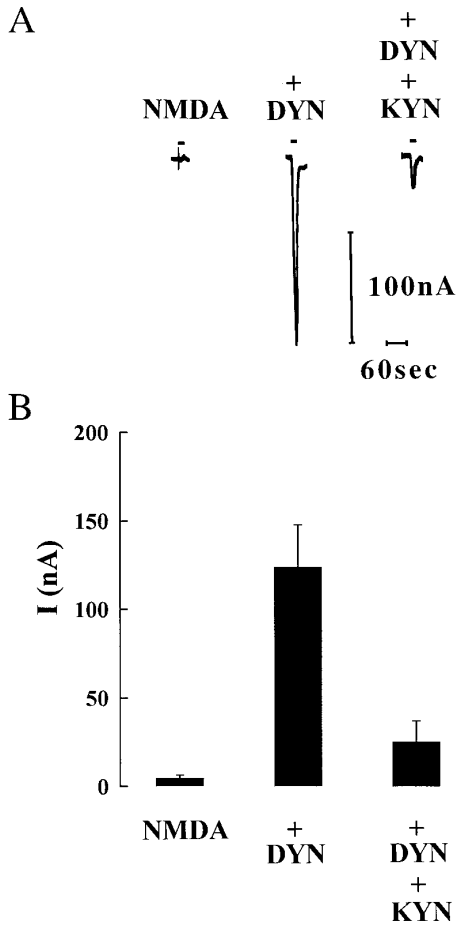

FIG. 5. Effect of kynurenic acid on potentiation of NMDA-activated current by dynorphin A(1–13) in presence of 100 nM added glycine. *A*: representative traces from oocyte expressing $\epsilon 4/\zeta 1$ NMDA receptor subunits of current activated by NMDA (*left trace*), NMDA plus 10 μM dynorphin A(1–13) (*middle trace*), and NMDA plus both 10 μM dynorphin A(1–13) and 50 μM kynurenic acid (*right trace*). *B*: bar graphs showing average (mean ± SE, $n = 4$) data relevant to traces in *A*.

of dynorphin A (Walker et al. 1982). In addition, in the presence of high glycine concentrations, dynorphin A(1–17) has been found to inhibit NMDA receptor-mediated responses more potently than does dynorphin A(2–17) (Chen et al. 1995). The second and third amino acids of the dynorphin A peptide are glycines. Because the potentiation of NMDA-activated current by dynorphin A(1–13) at low glycine concentrations appeared to be a glycinelike effect, we were interested to know whether the potentiation might involve either or both of these glycine residues. Figure 6 illustrates an experiment in which one or more of the first three amino acids were deleted from the dynorphin A(1–13) peptide to study this question. Figure 6*A* shows representative traces from one oocyte. At an extracellular added glycine concentration of 100 nM, the amplitude of NMDA-activated current was increased by 10 μM dynorphin A(1–13), (2–13), or (3–13); however, 10 μM dynorphin A(4–13), a peptide containing no glycine residue, did not increase the amplitude of NMDA-activated current. Figure 6*B* shows the average NMDA-activated current in these experiments. Potentiation of NMDA-activated current was significant for dynorphin A(1–13), (2–13), or (3–13) ($P < 0.01$, paired *t*-test), but not for dynorphin A(4–13) ($P > 0.05$, paired *t*-test). Dynorphin A(2–13) did not show a significantly greater potentiation of NMDA-activated current than dynorphin A(1–13) ($P > 0.05$, paired *t*-test); however, the potentiation by dynorphin A(3–13) was significantly greater than that of either dynorphin A(1–13) or dynorphin A(2–13) ($P < 0.05$, ANOVA). Dynorphin A(4–13) did not potentiate NMDA-activated current ($P > 0.05$, unpaired *t*-test).

Effect of dynorphin A(1–13) and (2–13) on NMDA-activated current in hippocampal neurons at low glycine concentrations

To determine whether dynorphin can also potentiate NMDA receptors on neurons, we investigated the effect of dynorphin A(1–13) and dynorphin A(2–13) on NMDA-mediated responses in hippocampal neurons at low extracellular glycine concentrations. Because the EC_{50} for glycine is very low for NMDA-activated currents in neurons (150 nM) (Johnson and Ascher 1992), the dynorphin experiments on hippocampal neurons were conducted in the absence of added glycine. Figure 7 shows that in the absence of added glycine, 10 μM dynorphin A(1–13) potentiated NMDA-activated currents by increasing the amplitude of the steady-state current. The observed potentiation of steady-state current, expressed as percentage of control, was 184 ± 30% ($n = 7$, $P < 0.05$, paired *t*-test). In hippocampal neurons,

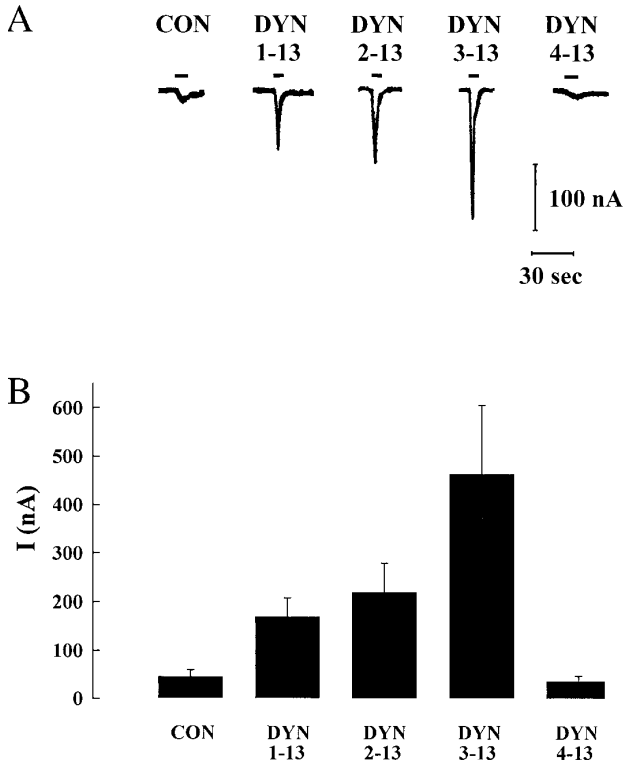

FIG. 6. Effects of dynorphin A(1–13), (2–13), (3–13), or (4–13) on NMDA-activated currents at low extracellular glycine concentrations. *A*: representative current traces comparing effect of dynorphin A(1–13), (2–13), (3–13), or (4–13) (each 10 μM) on NMDA-activated currents recorded from oocyte expressing $\epsilon 2/\zeta 1$ NMDA receptor subunits. Note that each of dynorphins, with exception of dynorphin A(4–13), potentiated NMDA-activated current. *B*: bar graphs showing average (mean ± SE) data relevant to traces in *A* ($n = 10$).

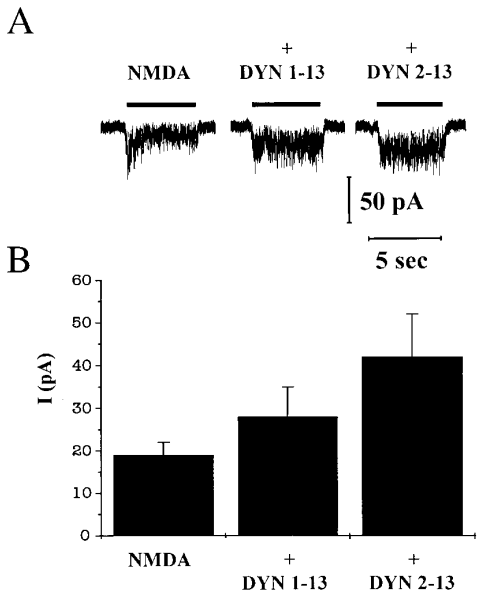

FIG. 7. Effect of dynorphin A(1–13) and (2–13) on NMDA-activated current in hippocampal neurons in absence of added glycine. *A*: representative traces showing, from *left* to *right*, current activated by 25 μM NMDA (*trace 1*) and current activated by this concentration of NMDA in presence of 10 μM dynorphin A(1–13) (*trace 2*) or 10 μM dynorphin A(2–13) (*trace 3*). *B*: bar graphs showing average (mean ± SE) data relevant to traces in *A* ($n = 5$).

10 μM dynorphin A(2–13) also potentiated steady-state NMDA-activated current (227 ± 37%, $n = 6$, $P < 0.0005$, paired *t*-test). There was no significant difference between the potentiation by dynorphin A(2–13) and that by dynorphin A(1–13) ($P > 0.05$, ANOVA).

Measurement of free glycine in experimental solutions

To address the possibility that the observed potentiation of NMDA responses by dynorphin at low extracellular glycine concentrations might be due to contamination of the experimental solutions by glycine, we measured the concentration of free glycine in various solutions used in these experiments. The bathing solution was found to have a free glycine concentration of 26 ± 4 nM ($n = 5$). Solutions containing 10 μM of the various dynorphin peptides had the following free glycine concentrations: dynorphin A(1–13), 30 ± 8 nM ($n = 4$); dynorphin A(2–13), 31 ± 4 nM ($n = 4$); dynorphin A(3–13), 27 ± 6 nM ($n = 4$); and dynorphin A(4–13), 36 ± 7 nM ($n = 4$). The free glycine concentrations of the bathing solution, and the bathing solution containing any of these dynorphin peptides, were not significantly different ($P > 0.05$, ANOVA).

DISCUSSION

The observations reported here show that, in the presence of low extracellular glycine concentrations, dynorphin A(1–13) can increase the amplitude of NMDA-activated current for both recombinant NMDA receptors expressed in *Xenopus* oocytes and native NMDA receptors on hippocampal neurons. The endogenously occurring dynorphin A(1–8), (1–17), and (1–32) also potentiated NMDA-activated current in *Xenopus* oocytes. Because dynorphin A is an endogenous κ-opioid agonist (Caudle and Isaac 1988; Chavkin et al. 1982), we tested whether the potentiation of NMDA-activated current by dynorphin A(1–13) is mediated by κ-opioid receptors. We found that the application of the κ-opioid agonist U50488 (10 μM) did not activate detectable current, nor did it affect the current activated by NMDA. Moreover, the opioid κ-receptor antagonist NorBN1 (1 μM) did not affect NMDA-activated current, nor did it alter the potentiation of NMDA-activated current by dynorphin A(1–13). These observations suggest that the dynorphin A(1–13) potentiation of NMDA-activated current does not involve κ-opioid receptors. In addition, the observation that dynorphin A(2–13) potentiated NMDA-activated current to an extent similar to the potentiation by dynorphin A(1–13) for both recombinant and native hippocampal NMDA receptors supports the interpretation that the dynorphin potentiation of NMDA-activated current does not involve κ-opioid receptors, because the first amino acid of the dynorphin peptide, tyrosine, has been found to be essential for the κ-opioid effect of dynorphin A (Walker et al. 1982).

NMDA receptor channels are gated by the combined action of the NMDA receptor agonist, such as NMDA or glutamate, and the coagonist glycine. It has been estimated that if there were no glycine present in the extracellular bathing solution, the NMDA receptor agonist alone would not gate the channel (Kleckner and Dingledine 1988). In view of this, the observation that at low extracellular glycine concentrations NMDA can activate significant NMDA receptor-mediated current in the presence of dynorphin A(1–13) is quite remarkable. In regard to this action of dynorphin A(1–13), the potentiation of NMDA-activated current by dynorphin A(1–13) was concentration dependent between 0.1 and 10 μM dynorphin A(1–13), with an EC_{50} value of 2.77 μM (Fig. 2). This compares with the glycine concentration-response curves, which were also concentration dependent between 0.1 and 10 μM, with EC_{50} values of 0.54–2.13 μM (Fig. 3*B*). The similarity between the dynorphin A(1–13) and the glycine potentiation of NMDA-activated current suggests that dynorphin A(1–13) has a glycinelike action on NMDA receptors.

Because glycine is the second and third amino acid in the dynorphin A peptide, it was important to determine whether the glycinelike effect of dynorphin A(1–13) was due to a glycine contamination of the dynorphin solutions. We therefore used HPLC to measure the free glycine concentration in the bathing solution in the absence and presence of dynorphin A(1–13), (2–13), (3–13), or (4–13). The observation that the free glycine concentration in these solutions was between 26 and 36 nM, and that there were no significant differences among these free glycine concentrations, indicates that glycine contamination does not explain the glycinelike action of dynorphin A(1–13), (2–13), and (3–13), because the dynorphin-free bathing solution and dynorphin A(4–13) had similar free glycine concentrations but did not exhibit a glycinelike action on NMDA-activated currents.

Because the biologically active part of the endogenous dynorphin A(1–17) consists of the first 13 amino acids, our findings with the synthetic, truncated dynorphin A(1–13), (2–13), (3–13), and (4–13) may be extrapolated to the endogenous dynorphin. Our observation that dynorphin

A(1–13), (2–13), and (3–13) show potentiation of NMDA-activated current and dynorphin A(4–13) does not may have implications for the physiological function of dynorphin A(1–17) and the biotransformation of this peptide. The endogenous dynorphin A(1–17) is processed very slowly, with a half-life of 3 h. The main transformation product is the nonopioid dynorphin A(2–17) (Chou et al. 1996). Dynorphin A(2–17) has a number of biological and pathophysiological activities, such as depression of firing rate of hippocampal neurons (Walker et al. 1982), inhibition of adenylyl cyclase (Claye et al. 1996), suppression of naloxone-induced opiate withdrawal (Takemori et al. 1993), and increase in natural killer cell activity (Bodner et al. 1992). Our data would suggest that potentiation of NMDA-activated current would be observed when the metabolites of dynorphin A retain at least one of the first three amino acids. Thus the first stages of biotransformation would not be expected to render the dynorphin A peptide ineffective in its nonopioid actions on NMDA responses. Dynorphin A(1–17) is further broken down to dynorphin A(3–17), which would be expected to be biologically active, and dynorphin A(4–17), which would not be expected to be biologically active.

The observation that dynorphin A(1–13) potentiation of NMDA-activated current was maximal at the lowest glycine concentrations (10 and 100 nM), and decreased in magnitude as glycine concentration was increased, suggests that the dynorphin A(1–13) potentiation of NMDA-activated current may be related to the action of glycine on NMDA receptors. One hypothesis that would explain the glycinelike action of dynorphin A(1–13) on NMDA receptors is that dynorphin A(1–13) potentiation of NMDA-activated current involves the glycine coagonist site on NMDA receptors. Consistent with this hypothesis is the observation that the glycinelike action was exhibited by dynorphin A(1–13), (2–13), and (3–13), which contain glycine as the second and/or third amino acid, whereas dynorphin A(4–13), which does not contain glycine as an amino acid, did not exhibit a glycinelike action on NMDA-activated current. Also consistent with the hypothesis that the dynorphin A(1–13) potentiation involves the glycine coagonist site on NMDA receptors is the observation that a competitive antagonist at the glycine coagonist site, kynurenic acid (50 μM), blocked the dynorphin A(1–13) potentiation of NMDA-activated current.

High-affinity glycine transporters have been reported in the nervous system (Guastella et al. 1992; Jursky and Nelson 1995; Smith et al. 1992). Moreover, one such transporter has been colocalized with NMDA receptors (Smith et al. 1992). If, as the result of the activity of such transporters, the glycine concentration in the synaptic cleft of glutamatergic synapses were to be low, for example $\leq$100 nM, the release of glutamate would activate little current at NMDA receptors. On the other hand, if dynorphin A were to be coreleased with glutamate, as for example at mossy fiber synapses on CA3 neurons in the hippocampus (Terrian et al. 1989), the action of dynorphin at NMDA receptors could greatly augment the response of these receptors to glutamate, resulting in NMDA receptor-mediated currents of significant amplitude. Thus the potentiation of NMDA receptor-mediated responses by dynorphin A could be an important physiological regulator of NMDA receptor function in the nervous system.

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Address for reprint requests: U. Brauneis, Laboratory of Molecular and Cellular Neurobiology, National Institute on Alcohol Abuse and Alcoholism, National Institutes of Health, 12501 Washington Ave., Rockville, MD 20852.

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